Reference: *Biol. Bull.* **189**: 91–105. (October/November, 1995)

The Incidence and Morphology of Subcuticular Bacteria in the Echinoderm Fauna of New Zealand

MAEVE S. KELLY[1], M. F. BARKER[2], J. DOUGLAS McKENZIE[1,*], AND JAN POWELL[3,‡]

[1]*The Scottish Association for Marine Science, PO Box 3, Oban, Argyll, Scotland,*
[2]*Departments of Marine Science and Zoology and* [3]*Department of Microbiology, University of Otago, Dunedin, New Zealand*

Abstract. New Zealand echinoderms (33 species drawn from all five extant classes) were examined for the presence of symbiotic bacteria by fluorescence and electron microscopy. Gram-negative, subcuticular bacteria (SCB) were found in 17 species from four classes. The SCB could be classified into two major morphological types. Some species had both types of SCB. The distribution of SCB was not obviously linked to host ecology but did appear to be related to host phylogeny. Related species usually all have SCB or all lack them. The number of SCB in five species was estimated to be between 8.41×10^8 and 4.96×10^9 g^{-1} ash-free dry weight of host tissue. Significant differences in bacterial load and relative proportions of the different types of bacteria were found among three congeneric echinoids (*Pseudechinus huttoni, P. albocinctus* and *P. novaezealandia*). *Ophiocoma bollonsi* was peculiar in having groups of bacteria enclosed in host cells (bacteriocytes) within the connective tissue of the tube feet.

Introduction

Symbiotic associations are increasingly seen as pathways for evolutionary innovation, allowing organisms to transcend the biochemical limitations of their own genome by harnessing the different biochemical capabilities of a symbiont (Margulis, 1981; Douglas, 1994). Examples of marine organisms utilizing symbionts to exploit otherwise closed energy sources include corals that use dinoflagellates to provide them with carbon fixed *via* photosynthesis (Muscatine *et al.*, 1984) and a variety of marine invertebrates that exploit the energy potential of free sulfide through the activities of sulfide-oxidizing bacteria (*e.g.*, Cavanaugh *et al.*, 1981). Symbioses between invertebrates and bacteria occur in protozoans (Fenchel *et al.*, 1977; Saffo, 1990); sponges (Vacelet and Donadey, 1977); cnidarians (Palincsar *et al.*, 1989); nematodes and turbellarians (Ott *et al.*, 1982); annelids (Giere, 1981; Hausmann, 1982); pogonophorans (Cavanaugh *et al.*, 1981); echiurans (Bosch, 1976); bivalve molluscs (Southward, 1986); cephalopods (McFall-Ngai, 1994); bryozoans (Lutaud, 1969); echinoderms (Holland and Nealson, 1978); tunicates (Mackie and Bone, 1978); and pterobranchs (Welsch, 1984). New symbioses are frequently reported (*e.g.*, Menon and Arp, 1993; Haszprunar *et al.*, in press). In some cases the biological role of the symbionts is obvious (*e.g.*, chemoautotrophic associations where the host lacks a gut), but in most it is enigmatic.

Echinoderms from all five extant classes are known to harbor symbiotic bacteria between the epidermal cells and the overlying layers of the cuticle (Holland and Nealson, 1978; Féral, 1980; McKenzie, 1987). These so-called subcuticular bacteria, or SCB (Holland and Nealson, 1978), have been recorded from Atlantic species of echinoderms (Holland and Nealson, 1978; Féral, 1980; McKenzie, 1987; Walker and Lesser, 1989; McKenzie and Kelly, 1994), from Australian crinoids (McKenzie, 1992), and from northeast Pacific ophiuroids (McKenzie and Kelly, 1994). The symbiosis is, therefore, geographically and phylogenetically widespread. Information on the exact distribution of SCB amongst echinoderms is, however, very vague. Some species are known to lack SCB (McKenzie and Kelly, 1994), and it may be possible to correlate the presence or absence of SCB with some common aspect of their hosts' biology if there is a large enough data set upon which to make such a comparison. The morphology of bacteria can also be useful in determining the probable trophic role of the symbionts. Likewise, a

Received 15 August 1994; accepted 25 July 1995.
* Author to whom correspondence should be addressed.
‡ Current address: Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, Maryland 21201.

high ratio of symbiont-to-host biomass may help identify particularly important associations. To this end we have been surveying a large number of echinoderm species from around the world for the presence, abundance, and morphology of SCB. This paper details the results of a study of 33 species of echinoderms that are found around New Zealand. Some preliminary results of this study were reported in Kelly *et al.* (in press).

Materials and Methods

Echinoderms were collected between October 1992 and January 1993, by beam trawl on the mid-shelf region off Otago harbor at a depth of 64 m (45°46.632′ 170°52.881′) and at a deeper water site of 120 m (45°44.539′ 171°01.197′), and by shore collections from the intertidal zone adjacent to Portobello Marine Laboratory. Some species were also collected from the intertidal zone at Matheson Rocks, north of Auckland, North Island. Animals were examined with epifluorescence light microscopy and tissues promptly fixed for transmission electron microscopy (TEM).

The epifluorescent microscopy was according to Hobbie *et al.* (1977) as adapted by Kelly and McKenzie (1992). A tube foot was removed, placed on a glass slide, stained with acridine orange at a concentration of 0.003% and gently squashed with the coverslip. This action frees many of the SCB from the tissue. Usually, 3–10 individuals of each species were examined for the presence of SCB. The bacterial load was quantified in three species of *Pseudechinus;* in *Asterodon miliaris, Ophiocoma bollonsi,* and *Amphipholis squamata;* and in juvenile *Pseudechinus huttoni* (diameters of 14–18 mm). Rods and spirals were counted separately for the three species of *Pseudechinus* and for the juvenile *P. huttoni.*

For direct counts of bacterial numbers, larger pieces of tissue were removed from the seven species mentioned above and homogenized with either a hand-held glass tissue grinder or an Ultraturrex mechanical tissue homogenizer. For ophiuroids, a piece of arm was used; for echinoids, a portion of test was taken from the ambulacral groove area. Care was taken not to rupture the gut as the test was removed. The tissue was homogenized at a ratio of 1 g tissue: 2 ml of filtered (0.1 μm) seawater and then mixed with an equal volume of acridine orange. An exact volume of the homogenate (5 μl) was slide-mounted using No. 1 22 × 22 mm coverslips. In each homogenate, all the bacteria observed within an eyepiece-mounted Whipple grid (at 1000× magnification) were counted from 20 randomly selected areas. The whole process was repeated twice for each of 10 animals. Because individual *Amphipholis squamata* are small, homogenates were made from pooled individuals (one of 8 and one of 11 individuals). To check the accuracy of the counting technique, 10 counts were made from one homogenate of *P. huttoni* test. External or contaminant bacteria, which were only rarely seen, differed obviously in size and appearance from the SCB. The number of SCB was expressed per gram of tissue wet weight and per gram of ash-free dry weight, to allow a comparison between species with different ratios of soft tissue to skeletal calcite. For wet weights, tissue samples were rinsed in filtered (0.1 μm) seawater, shaken to remove excess surface water, and then weighed. For dry weights, tissues were dried at 40°C until they reached a constant weight. Ash weights were obtained after tissues were incinerated in a muffle furnace at 400°C. Ash-free dry weights were obtained by subtracting the ash weight from the dry weight for each sample. Microscopical observations and counts of the bacteria were made with an Olympus Vanox epifluorescence microscope.

In preparation for TEM, tissues from three individuals of each species (where available) were fixed in 4% glutaraldehyde in 0.1 *M* cacodylate buffer, rinsed in fresh buffer and decalcified, if necessary, in saturated EDTA. Postfixation was with 1% osmium tetroxide in filtered seawater, followed by dehydration in ethanol and embedding in Agar 100 resin. Silver sections were cut on an LKB III microtome, stained with ethanoic uranyl acetate and aqueous Reynold's lead citrate, and examined on a JEOL 100S at 60 Kv.

Attempts were made to culture symbionts from the ophiuroids *A. squamata* and *Ophiocoma bollonsi* and the echinoid *Pseudechinus huttoni.* The experimental procedures for *A. squamata* were (a) that of Walker and Lesser (1989)—surface sterilization of whole animals in 70% isopropyl alcohol for 30 s followed by two rinses in 75% sterile artificial seawater (ASW) prior to homogenization in sterile glassware and plating on agar; (b) a modified procedure (Lesser, pers. comm) as above but with surface sterilization time reduced to 5 s; and (c) an alternative method using protocol *b* but separating the arms from the disk of the animals before homogenization. For *O. bollonsi,* only tissue from the arm was used; a portion of the arm was surface sterilized and then the tube feet were removed and homogenized. The homogenate was then spread on plates or used to inoculate liquid culture medium. For *P. huttoni,* small squares of test from the ambulacral groove were surface sterilized and treated as above. Animals that had not been subjected to the surface sterilization procedures were used as controls. The brittlestar *Ophionereis fasciata,* which does not have SCB, was used as a further control. The tube feet or portions of tissue were placed in a watch glass and ground with a glass grinder. The glassware was surface sterilized with 70% ethanol. Marine broth (Difco, 300 μl) was used as a diluent.

Ultraviolet (UV) exposure and repeated washing in autoclaved filtered seawater were investigated as alternative methods for surface sterilization. Tube feet from *O. bollonsi* and arms from *A. squamata* were exposed to UV

for 2, 5, 10, and 15 min prior to homogenization in 300 μl diluent. Whole *A. squamata* and squares of the test of *P. huttoni* were washed up to five times in autoclaved filtered seawater. Homogenates were made of the *A. squamata* arms and of the squares of urchin test. The homogenates were prepared in 300 l diluent, then plated on agar and inoculated in broth as outlined below. Equivalent untreated tissues were used for controls.

The medium used was Zobell's modified 2216E prepared with 75% (w/v) ASW. Salt solutions (1 *M*) for the ASW were prepared and autoclaved. The agar was then prepared, the yeast and peptone were added, and the agar was autoclaved and held at 56°C. To prevent precipitation of the sterile salt solutions, they were warmed and added to the agar before the plates were poured. To determine the number of colony-forming units (CFU) per animal, logarithmic dilutions were prepared in marine broth and spread plated. The dilution broths were also cultured as enrichment for slow-growing or stressed organisms unable to grow initially on solid medium. The plates were incubated at room temperature (18–21°C) and examined daily for bacterial growth. After 48 h, the resulting bacterial colonies were counted and CFU per sample were determined. The plates were then maintained for up to 21 days to observe the appearance of any slower growing colonies.

Results

General observations

Table I shows the number of species within each class that were examined and the number and percentage that were found to harbor SCB. Of the 33 species of New Zealand echinoderms examined, 17 had SCB (Table II). When the SCB load was substantial, the bacteria were easy to find with epifluorescence. However, in some cases, TEM examination demonstrated SCB that had escaped detection by epifluorescence.

Table I

Number of species examined and number and percentage with SCB

Class	Number of species	Number (%) with SCB
Echinoidea	7	5 (71)
Ophiuroidea	9	5 (56)
Asteroidea	14	5 (36)
Holothuroidea	2	2 (100)
Crinoidea	1	0 (0)
Total	33	17 (51.5)

Bacterial morphology

In all of the echinoderms examined, the SCB appeared to be gram-negative as they had two membranes (Figs. 4, 11) surrounding the cytosol (Neidhart *et al.*, 1990). The periplasmic layer between the two membranes was usually thin and homogeneous. None of the bacteria appeared flagellated, nor were pili observed. The cytosol usually evenly surrounded the nucleoid area and was usually rather homogeneous, with no evidence of either internal membranes (other than membrane-bound vacuoles) or granular inclusions. Round spaces in the cytosol, indicating where material had been lost during processing, were not infrequent and may be poly-β-hydroxybutyrate storage areas (Berkeley, 1979). The DNA in the nucleoid was often condensed into an electron-dense lobulated structure (Fig. 3), but in some SCB it was much more diffuse (Fig. 5).

The SCB from the New Zealand species of echinoderms were more variable in their morphology than those previously encountered. McKenzie and Kelly (1994) described three morphological types of bacteria from ophiuroids (Types 1–3). Type 1 SCB are short rods that characteristically occur as paired bacteria within a single, complex capsule. This type has been found only in species of *Ophiothrix*, although another brittlestar, *Ophiopholis aculeata*, has SCB that are intermediate between Type 1 and Type 3 SCB. SCB of Type 2 were the most common. They are long, thin (0.1–0.2 μm), often electron-dense rods with little evident ultrastructure. They lack capsules and rarely have vacuoles. They are usually spirals, but vary from perfectly straight rods through spirals with long wave-lengths (Figs. 5, 7A) to tightly kinked spirals with short wave-lengths (Fig. 9). Type 3 SCB are straight, broad (0.2–0.5 μm) rods, often with capsules and characteristically with vacuoles, though these are not always present. The New Zealand species could be further categorized into three subdivisions of Type 3 SCB. The first subtype is the "classic" Type 3 as described by McKenzie and Kelly (1994). The second subtype differs in having far more vacuoles than normally encountered in Type 3 SCB. This subtype was found in *Ophiomyxa brevimira* (Fig. 10) and *Stichopus mollis* (Fig. 2). The third subtype was found in the three species of *Pseudechinus*. It was a straight, baton-shaped rod with a well-defined, granular periplasmic layer and no obvious vacuoles (Fig. 3).

In some species, more than one type of SCB was found within a single host. Fixation artifact or pathology can, however, result in Type 2 SCB resembling Type 3 SCB, and care has to be taken not to confuse such artifacts with genuine Type 3 SCB. Bacteria appear to swell in fixation, causing the membranes to become more distinct and space to appear around the chromatin (Fig. 7B). The shape of the bacterium can become more rounded and eventually irregular. In severe cases, the chromatin is isolated in the center of the bacterium and the cell membranes are greatly disrupted. Such bacteria can be mistaken for poorly fixed host microvilli or blebbed pieces of epidermal tissue.

Table II

Observation and description of SCB as seen under epifluorescent (EF) and transmission electron microscopy (TEM)

CLASS Order Family	Species and authority*	SCB EF/TEM†	SCB Description‡
CRINOIDEA			
Comatulida			
Comasteridae	*Comanthus novazealandiae*[2] Clark	n/n	Some bacteria found on surface not SCB, TEM only.
HOLOTHUROIDEA			
Aspidochirotida			
Stichopodidae	*Stichopus mollis* (Hutton)	n/y	Short rods, T3.
Apodida			
Chiridotidae	*Trochodota dunedinensis* (Parker)	n/y	Short, electron dense rods, T?
ECHINOIDEA			
Cidaroida			
Cidaroidae	*Goniocidaris umbraculum*[3] (Hutton)	y/y	Short rods, 1–2 μm. T3.
Echinacea			
Temnopleuridae	*Pseudechinus huttoni* Benham	y/y	Rods 2–3 μm and spirals, spirals can be >10 μm, T2 & T3.
	Pseudechinus novaezealandiae (Mortensen)	y/y	Rods and spirals, spirals can be >10 μm, T2 & T3.
	Pseudechinus albocinctus (Hutton)	y/y	Rods and spirals, spirals can be >10 μm, T2 & T3.
Echinometridae	*Evechinus chloroticus* (Valenciennes)	n/n	
Clypeasteroida			
Arachnoidae	*Fellaster zelandiae*[3N] (Gray)	y/y	Spirals, difficult to find. T2.
Spatangoida			
Loveniidae	*Echinocardium cordatum*[N] (Pennant)	n/–	TEM specimens poorly fixed.
ASTEROIDEA			
Paxillosida			
Asteropectinidae	*Astropecten primigenius* (Mortensen)	y/y	Short rods, 2–3 μm and spirals. T2.
	Astropecten polyacanthus[1] Muller & Troschel	n/n	Cuticle not preserved.
Valvatida			
Asterinidae	*Patiriella regularis* Verrill	y/y	Spirals 5–8 μm. T2.
Goniasteridae	*Pentagonaster pulchellus* Gray	n/–	
Odontasteridae	*Asterodon miliaris* (Gray)	y/y	Rods 3 μm, sometimes in short chains of 2–3. Spirals seen only with TEM. T2 & T3.
	Odontaster benhami (Mortensen)	y/y	Straight rods, 10 μm and spirals 10 μm. T2 & T3.
Ophidiasteridae	*Ophidiaster kermadecensis*[1N]	n/y	Rods with vacuoles and coats, T3?
Spinulosida			
Echinasteridae	*Henricia ralphae* Fell	n/n	
Forcipulatida			
Asteriidae	*Sclerasterias mollis* (Hutton)	n/n	
	Coscinasterias calamaria (Gray)	n/n	
	Allostichaster insignis (Farquhar)	n/n	
	Allostichaster polyplax (Muller & Troschel)	n/n	
	Astrostole scabra[1] (Hutton)	n/n	
	Calvasterias suteri (de Loriol)	n/–	
OPHIUROIDEA			
Phrynophiurida			
Asteroschematidae	*Astrobrachion constrictum*[2] (Farquhar)	n/y	Spirals, T2.
Ophiomyxidae	*Ophiomyxa brevimura* Clark	y/y	Short rods, occasional spirals. Only T3 with TEM.
Ophiurida			
Amphiuridae	*Amphipholis squamata* Delle Chiaje	y/y	Long rods 3–4 μm, shorter fat rods (2 μm) often in chains of 2. T3.
	Amphiura abernethyi[1] Fell	y/–	Rods 4–5 μm EF only.
	Amphiura amokurae Mortensen	y/y	Spirals 6–8 μm, T2. Unusually broad: 0.25–0.30 μm.
Ophiocomidae	*Ophiocoma bollonsi* Farquhar	y/B	5 μm thin rods seen with EF, sometimes faint, bacteriocytes with TEM.
	Ophiopteris antipodum Smith	y/n	5 μm rods seen with EF, sometimes faint.
Ophiodermatidae	*Pectinura maculata* (Verill)	n/n	
Ophionereidae	*Ophionereis fasciata* (Hutton)	n/n	

* 1, 2, or 3 = number of specimens available if 3 or less; N = collected from North Island

† y = seen; n = not seen; – = no sample; B = bacteriocyte.

‡ T2 = Type 2 SCB; T3 = Type 3 SCB. Lengths are from epifluorescence measurements.

Occurrence within class

Crinoidea: Examination of the pinnules of *Comanthus novaezealandiae* by epifluorescent microscopy revealed no evidence of SCB. A few large bacteria were found in TEM sections, but these were outside the cuticle and were not considered to be invasive or to be any type of SCB. The same type of bacteria were found in both specimens examined, collected at the same time and location.

Holothuroidea: The apodous holothurian *Trochodota dunedinensis* appeared to have an unusual form of SCB

that could not be easily classified as belonging to any of the major types of SCB (Fig. 1). These were uncommon and had an electron-dense, filamentous appearance. SCB of a similar morphology have been found in *Labidoplax digitata*, another apodous holothurian (Kelly and McKenzie, 1995). SCB were commonly present in TEM sections of the aspidochirote holothurian *Stichopus mollis*, although none had been found previously with epifluorescence. These short rods had numerous vacuoles (Fig. 2).

Echinoidea: SCB, in the form of short rods, were found in *Goniocidaris umbraculum*, a representative of the primitive subclass Perischoechinoidea. SCB were easily found by epifluorescence in all the specimens that were examined. TEM fixation of both the host cytoplasm and bacteria was poor, but the symbionts appeared to be Type 3 SCB, with internal vacuoles. The material in these vacuoles was often lost, forming a hole in the section.

SCB were found in all three species of the genus *Pseudechinus*. Each had two distinct morphological types of bacteria: straight, baton-shaped rods (Fig. 3) and a typical Type 2 spiral form (Fig. 4). Both forms could be seen with epifluorescence and TEM. The SCB were found lying both below and within the fibrous layer of the cuticle. In both types of SCB the chromatin was usually condensed into thick, electron-dense fibers running up the center of the bacteria. The baton-shaped rods had the appearance of being rigid and had rounded caps at either end of the baton (Fig. 3), rather than tapering at their ends. The most striking feature of this type of SCB was the well-defined, granular periplasmic space. The three species of *Pseudechinus* were collected throughout the austral summer months, and SCB were always present regardless of the reproductive condition or size of the host. The smallest *P. huttoni* collected had a diameter of 14 mm and was probably less than 2 years old.

Detection of SCB by epifluorescence microscopy of tube feet from the sand dollar *Fellaster zelandiae* was difficult. A few irregular rods were seen, but the fluorescence faded unusually quickly. The same dampening effect has been noted when examining some other echinoids (for example, *Evechinus chloroticus*) and some darkly pigmented ophiuroids. TEM showed that the symbionts in *F. zelandiae* were spiral Type 2 SCB, though few were seen.

No SCB were found in specimens of *Echinocardium cordatum* with either technique. None of the specimens of *E. cordatum* fixed for TEM had retained their cuticles, and their morphology was also poorly preserved.

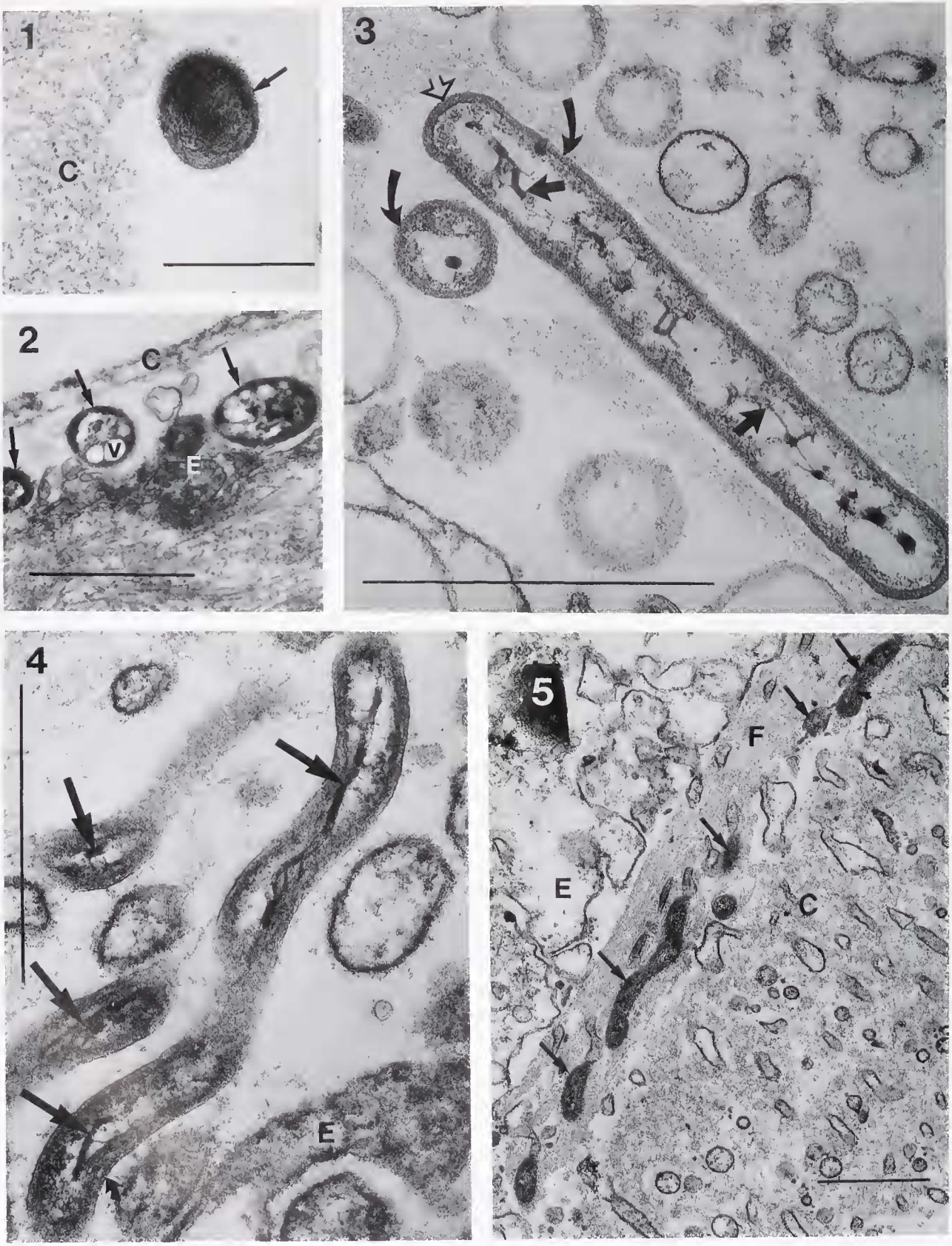
Asteroidea: With epifluorescence, SCB were difficult to find in *Astropecten primigenius*. In only one specimen were a few rods and spirals observed. In contrast, TEM of this species revealed that typical Type 2 SCB were common in the fibrous part of the cuticle (Fig. 5), where they sometimes appeared to be aligned parallel to the fibers of the lower cuticle. Larger, misshapen bacteria, which were probably distorted Type 2 SCB, were also seen. No SCB were found in the single specimen of *A. polyacanthus* from Auckland. The cuticle was not intact in this specimen.

In the cushion star *Patiriella regularis*, abundant spiral-shaped SCB with little internal specialization were found with epifluorescence, and TEM revealed Type 2 SCB in the lower, fibrous part of the cuticle. *Asterodon miliaris* and *Odontaster benhami* are biscuit stars that are extremely difficult to tell apart, being identifiable by the number of projections on the oral plates (Fell, 1962). Even so, the two species were readily distinguished on the basis of their SCB. With epifluorescent microscopy (Fig. 6A), *A. miliaris* had mostly straight, brightly fluorescing rods, forming short chains of 2–3 cells; whereas *O. benhami* had a mixture of two morphological types. TEM showed, however, that *A. miliaris* did have both Type 3 and Type 2 SCB, though the latter were much less common than they were in *O. benhami*. The Type 3 SCB in *O. benhami* were often dumb-bell shaped (Fig. 6B). In the one specimen of *Ophidiaster kermadecensis* that was examined, no SCB were found with epifluorescence microscopy. A few Type 3 rods with vacuoles and well-developed capsules were found amongst the microvilli in TEM sections. These were probably SCB but, as the specimen lacked its cuticle, this requires confirmation. The remaining seastar of the Order Valvatida, *Pentagonaster pulchellus*, was examined only by epifluorescent microscopy and no SCB were noted.

No SCB were found in *Henricia* or in any of the seastars belonging to the order Forcipulatida with either epifluorescence or TEM. Rod-shaped bacteria were commonly found in TEM sections of a single specimen of *Calvasterias suteri;* however, the tissues were considerably disrupted and bacteria were found throughout the sections, including in connective tissue. These bacteria are probably not SCB and may be pathogenic, but further study will be necessary to confirm this. A single bacterium was found embedded in an indentation of the outermost layer of the cuticle of a specimen of *Sclerasterias mollis*. This was not thought to be an SCB.

Ophiuroidea: Many SCB were found in TEM examination of decalcified arm tips from *Astrobrachion constrictum* (Fig. 7A). They were spirals but were occasionally seen to vary in cross-sectional area (Fig. 7B). The larger types are probably an artifact resulting from the swelling of the more commonly seen form. However, no bacteria were found when the tube feet were examined with epifluorescence. The small amount of soft tissue on the arms and the small tube feet make epifluorescent examination of tissue squashes difficult, whereas the SCB are found more easily with TEM after decalcification.

Numerous thin, rod-shaped bacteria were found in tube-foot squash preparations from *Ophiocoma bollonsi* and *Ophiopteris antipodum*. The fluorescence faded very rapidly in homogenates of the tissue of these species,



Figures 1–5. Scale bars represent 1 μm.

making counting difficult. Despite intensive searching with TEM, no SCB were ever found lying in the subcuticular space of either species, although the bacteria were always found in tube foot squash preparations with epifluorescent microscopy. This initially led to the assumption that the bacteria were in discrete areas of the subcuticular space and might have been missed during TEM examination. However, large bacteriocytes packed with long, spiral-shaped bacteria were later found in the connective tissue of *O. bollonsi* (Fig. 8). The bacteriocytes were about 10 μm diameter in TEM micrographs. Occasionally the spiral bacteria were seen free in the tissue. *Ophiomyxa brevimira* had a mixture of morphological types of SCB: short rods, often forming chains of two; longer rods; and a few spiral forms were seen with epifluorescence. With TEM, short, heavily vacuolated Type 3 rods were found to be the predominant type. Holes often formed in the sections where there were vacuoles (Fig. 10).

Two species of *Amphiura* were examined. *A. amokurae* was very common under stones along the beach adjacent to the Portobello laboratory. One specimen of *A. abernethyi* was obtained by trawl from a depth of 120 m outside Otago Harbor. *A. amokurae* had long, tightly bound, spiral SCB that were obvious in both epifluorescence and electron microscopy (Fig. 9). These had an unusually large diameter for Type 2 SCB. In *A. abernethyi*, straight rods, 4–5 μm long, were seen with epifluorescence, but the only available specimen was in poor condition when fixed for TEM, and no cuticle or bacteria could be found around the surface. *Amphipholis squamata* had long, vacuolated, rod-shaped bacteria (Fig. 11). These were very common and were easily seen with both electron and light microscopy.

Quantification

The results of the quantification of bacterial load for all three species of *Pseudechinus*, *Asterodon miliaris*, *Ophiocoma bollonsi*, *Amphipholis squamata*, and juvenile *Pseudechinus huttoni* (test diameters of 14–18 mm) are given in Table III. No significant differences were found between arms or ambulacral areas from the same individual animal for any of the species (Student's *t* and Wilcoxon signed rank test). To measure the variability of the counting technique, 10 counts were made from one homogenate of *P. huttoni* test. The mean count per 20 fields of view was 17.9, with a standard deviation of 4.63. Rods and spirals were also counted separately for the three species of *Pseudechinus* and for juvenile *P. huttoni*. A one-way analysis of variance (ANOVA) and a Fisher's least significant difference test (LSD) was then applied to the data (Table IV). *P. huttoni* adults and juveniles both had greater numbers of rods than spirals ($P < 0.05$). The other two species had more spirals than rods, though these differences were not significant. As can be seen from Table IV, there are numerous significant differences between the species in their relative numbers of rods and spirals.

Culturing

The methods described in Walker and Lesser (1989) and Lesser and Blakemore (1990) (*i.e.*, plating the homogenates of *A. squamata* following a surface sterilization in 70% isopropyl alcohol for 30 s), failed to produce bacterial colonies on agar plates. However, when whole animals were treated for only 5 s, prior to separate homogenization of arms and disks, bacterial colonies grew on plates inoculated from homogenates of the disks (1.5×10^2 mixed CFU/disk) but not from the arms. This compares with 2.6×10^4 and 2.6×10^3 CFU for control (not surface sterilized) arms and disks respectively. Although the plates were examined daily, no fresh colonies appeared in a 10–14 day period as described by Lesser and Blakemore (1990) or after 14–21 days as described by Lesser (pers. comm. to MSK).

The other species produced results similar to those obtained from *A. squamata*. The surface sterilization for 5 s usually resulted in no colony formation on plates, but nonsterilized animals produced colonies regardless of whether they contained SCB (*P. huttoni*) or not (*Ophiocoma bollonsi* and *Ophionereis fasciata*). In the few cases

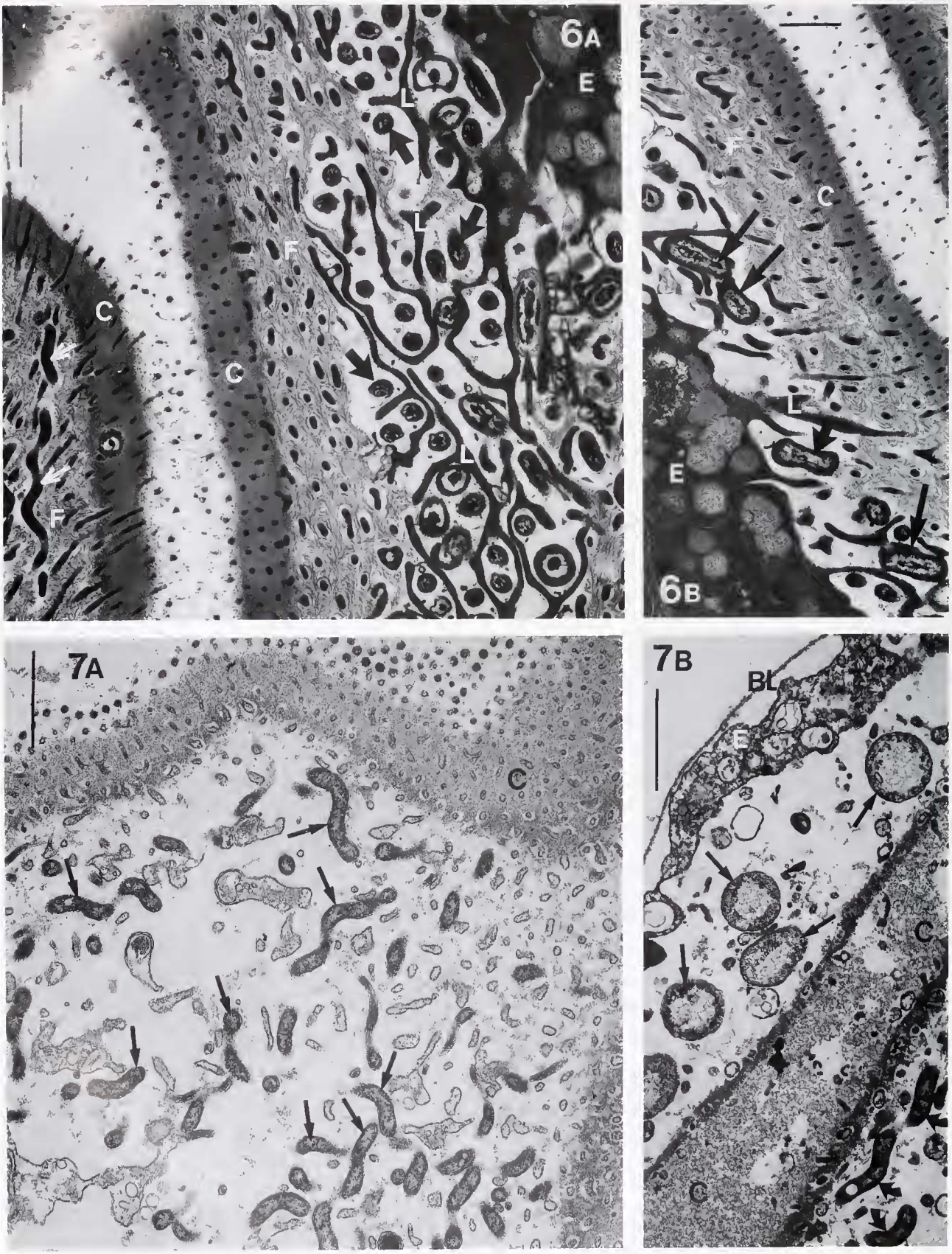
Figure 1. Transverse section of SCB (arrow) from *Trochodota dunedinensis* body wall, lying beneath the cuticle (C) of the body wall. SCB coat has fibrous appearance.

Figure 2. Transverse sections through Type 3 SCB (arrows) beneath the cuticle (C) of *Stichopus mollis* tube foot. SCB have numerous vacuoles (v). E, epidermal support cell.

Figure 3. Baton-shaped SCB from *Pseudechinus huttoni* tube foot, longitudinal section and transverse section. The chromatin is condensed into electron-dense fibers running along the center of the bacterium (arrow). Note granular appearance of periplasmic space (curved arrow) and rounded cap-like ends (open arrow).

Figure 4. Type 2 spiral form SCB from *Pseudechinus huttoni* tube foot, showing condensed chromatin fibers (arrows) and double membranes characteristic of Gram negative bacterium (curved arrow). E, epidermal support cell.

Figure 5. Typical Type 2 SCB (arrows) situated in the lower fibrous part of the cuticle (F) in *Astropecten primigenius* tube foot. SCB sometimes appear aligned with the fibers of the lower cuticle. C, outer layer of cuticle; E, epidermal support cells.



Figures 6–7. Scale bars represent 1 μm.

in which colonies did form, these were always of more than one type and of similar morphology to the colonies growing on control plates.

The UV treatments reduced the number of bacterial colonies from the *A. squamata* arms but did not totally eliminate the bacteria, even at longer exposure times. The resulting bacterial colonies were of a variety of morphological types and were similar to those on the control agar plates. Few colonies were isolated from the nonsterilized tube-foot homogenates of *O. bollonsi* or from those given a short UV exposure; no bacteria were cultured from tube feet exposed to UV for longer time periods. Repeated washing with sterile ASW had no apparent effect in reducing the number of colony-forming bacteria associated with the echinoderms tested. Whenever bacteria were cultured, the variety of colony morphologies was similar to that of control animals.

Discussion

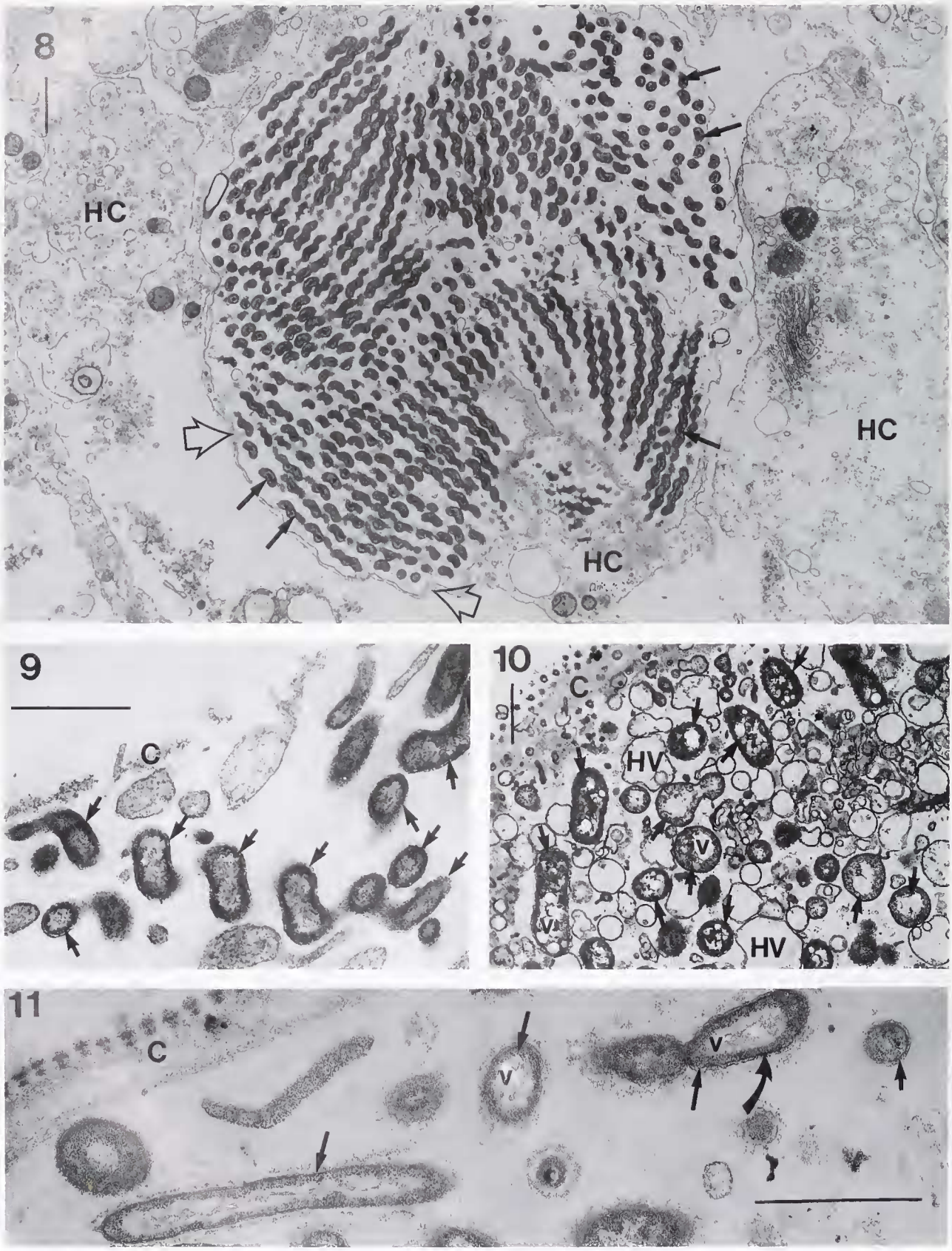
About half of the echinoderm species we examined from New Zealand contained SCB. In a survey of 63 species of echinoderms from the shelf seas around the British Isles, more than 60% had SCB (Kelly and McKenzie, in press). These surveys and most of the other information on SCB distribution have been from temperate, shallow-water echinoderm faunas. The distribution of SCB in tropical and polar echinoderms is unknown, though McKenzie (1992) described SCB in crinoids from the Great Barrier Reef, Australia. SCB have also been found in deep-sea holothurians (Roberts *et al.*, 1991) and deep-sea ophiuroids (unpub. obs.). There is no obvious correlation between the occurrence of SCB and the habitat, feeding strategy, or other ecological aspects of the host. The New Zealand echinoderms were collected from a range of habitats, including sandy beaches, rocky shores, and depths of 60 m and 120 m. SCB were found in animals of different reproductive condition and size or age. Again, the number of SCB within a species was apparently not linked to host ecology. Similar conclusions were reached in other studies (McKenzie and Kelly, 1994; Kelly and McKenzie, in press). SCB are thus a general, though not universal, phenomenon of echinoderms rather than being found only in particular ecological groupings of species.

One factor that does relate to SCB distribution is host phylogeny. The following generalities are supported by the present observations and those of earlier studies. (1) When SCB are recorded from a species, then all individuals of that species will have symbionts; (2) species congeneric with a symbiont-containing species will all have SCB; and (3) co-familial species will probably all have or all lack SCB (McKenzie and Kelly, 1994; Kelly and McKenzie, in press). Every individual of well-studied species, such as the three *Pseudechinus* species, had SCB. *Amphipholis squamata* from New Zealand (this study), North America (Walker and Lesser, 1989) and North Europe (McKenzie and Kelly, 1994) all appear to have morphologically identical SCB. It would be interesting to check the molecular similarities between the hosts and between the symbionts from these separate regions. The presence of SCB in all three species of *Pseudechinus* supports the second generality; this is also true for the brittlestar genus *Amphiura*. SCB were present in both New Zealand species and have also been found in three European species of *Amphiura* (Kelly and McKenzie, in press). SCB have been recorded from *Astropecten irregularis* and three species of *Echinocardium* (Holland and Nealson, 1978; Kelly and McKenzie in press). The failure to demonstrate SCB in the New Zealand species *Astropecten polyacanthus* and *Echinocardium cordatum* was probably due to the poor cuticular fixation in all the specimens examined; further investigations of these species may reveal their presence. Three Australian species of the feather star genus *Comanthus* (*C. timorensis*, *C. parvicirrus*, and *C. alternans*) were examined by McKenzie (1992). None had SCB, as was the case for the New Zealand *C. novozealandiae*. Examples of co-familial species either having or lacking SCB are best seen in the asteroids. *Asterodon* and *Odontaster* both have SCB. *Patiriella* belongs to the Asterinidae and SCB have also been reported from other species in this family (Cameron and Holland, 1983; Souza Santos and Sasso, 1970; Kelly and McKenzie, in press). None of the species belonging to the family Asteriidae (*Sclerasterias*, *Coscinasterias*, *Allostichaster*, *Astrostole* and *Calvasterias*) had SCB. The earlier report of *Calvasterias* having SCB (Kelly *et al.*, 1994) was erroneous.

McKenzie and Kelly (1994) noted a correlation between SCB morphology and host phylogeny in ophiuroids. Species within a genus usually all have similar SCB, and this is sometimes also true of co-familial species. This correlation is partially supported by the results from the New Zealand species, but there are exceptions. In specimens

Figure 6. (A) Mixed morphological types of SCB in *Odontaster benhami* tube foot. Type 2 SCB (white arrows) in the lower fibrous layer (F) of the cuticle (C) and Type 3 SCB mostly in transverse section (arrows). L, lamellae of support cells; E, epidermal support cell. (B) Dumbbell-shaped Type 3 SCB (arrows) lying below the cuticle (C). L, lamellae of support cells; E, epidermal support cell.

Figure 7. (A) Numerous Type 2 SCB (arrows) lying beneath the cuticle (C) of *Astrobrachion constrictum* tube foot. BL, basal lamima. (B) Two opposing surfaces of tube foot from *Astrobrachion constrictum* (C). On the upper left the SCB in transverse section appear enlarged and distorted (arrows). On the lower right the SCB appear as typical Type 2 SCB (curved arrows). E, epidermal support cell.



Figures 8–11. Scale bars represent 1 μm.

of *Amphiura filiformis* and *A. chiajei* collected from the British Isles (McKenzie and Kelly, 1994), the SCB are Type 3 rods, classically with membrane-bound vacuoles tearing to form holes in the sections. Spiral forms have occasionally been seen with the epifluorescent microscope but not so far with TEM, the straight rods being much more numerous. McKenzie and Kelly (1994) found a single Type 2 SCB in another amphiurid (*Microphiopholis atra*). *A. amokurae* is apparently unusual amongst amphiurids in having a predominance of Type 2 SCB instead of the Type 3 that might have been predicted. The fact that some species have two types of SCB, combined with the low overall level of variation in SCB morphology, limits the usefulness of bacterial morphology as a character in investigating possible co-evolution between the symbionts and their hosts.

It is not known if the different morphological types of SCB represent separate genotypes. Other symbioses show evidence that bacteria with different morphologies have different genetic identities. Among homopteran insects, 55% of the species are thought to contain more than one type of symbiont (Buchner, 1966), and both sulfide-oxidizing and methylotrophic symbionts have been found within the same bivalve host (Fisher *et al.*, 1993). The fact that the three *Pseudechinus* species (Table IV) and *Asterodon miliaris* and *Odontaster benhami* consistently differ in the ratio of the SCB types within them may indicate that the morphotypes reflect different bacterial genotypes. Alternatively, this difference could result from a single SCB genotype having morphological plasticity within its echinoderm host. The ability of a single genotype to exhibit considerable pleomorphism in response to subtle environmental variations is known in many bacteria (Berkeley, 1979) and has been recorded in symbioses such as some mycetocyte-insect associations (Houk and Griffiths, 1980; Smith and Douglas, 1987). The presence of both major types of SCB is phylogenetically widespread, and it may be that they are both potentially present in all species. This would strengthen the argument that the types are phenotypic variants rather than different genotypes. Molecular investigations of 16S rRNA variation within the symbionts of single species could be the best way of resolving question.

The estimates of bacterial loading in New Zealand's echinoderms were similar to those recorded for ophiuroids from the British Isles. McKenzie and Kelly (1994) estimated a bacterial load of 4.60×10^9 g^{-1} AFDW for *Amphipholis squamata* collected from the west coast of Scotland. This compares to 4.96×10^9 g^{-1} AFDW for *A. squamata* from New Zealand. The figure for *A. squamata* given by Lesser and Blakemore (1990) is not directly comparable; it relates to bacterial colonies per animal rather than to SCB g^{-1} AFDW. *Pseudechinus huttoni* had a high bacterial loading, only slightly lower than that recorded from *A. squamata* and considerably higher than that of the other two species of *Pseudechinus*. The three species of *Pseudechinus* are broadly sympatric subtidally on the Otago coast and maintain genetic isolation from each other, although they can be readily hybridized in the laboratory. Color is the most obvious character distinguishing the species, although there are other morphological differences such as test thickness and length of spines. The differences in overall bacterial load and in the ratios of the types of SCB present therefore warrant further investigation.

Few comparative figures are available from other symbioses. The density of SCB in all the species is similar to the bacterial loading in the trophosome of vestimentiferans (up to 10^9 bacteria g^{-1} wwt—Cavanaugh *et al.*, 1981; Powell and Somero, 1983). The echinoderm symbionts clearly have the potential to be metabolically important to their hosts. However, the trophosome is a much higher proportion of the total body mass of pogonophorans than the integumental tissues are in echinoderms, so any contribution of SCB to their hosts' holistic energy budget is likely to be proportionately lower.

Walker and Lesser (1989) claimed to have cultured SCB from *Amphipholis squamata*, which they identified as an undescribed species of *Vibrio*. Attempts to reproduce this result from New Zealand *A. squamata* and from other symbiont-containing echinoderms (this study and unpublished observations from Scottish echinoderms) did not produce any evidence of a symbiont in culture. It is difficult to see how any SCB could have survived the sterilization described in Lesser and Blakemore (1990). Even a 5-s exposure to isopropyl alcohol killed the host, so 30 s of exposure would have penetrated the cuticle and presumably killed the SCB. In our experiments, bacteria never grew when the animals had more than a 5-s exposure to isopropyl alcohol. Even those that occasionally grew at an exposure of 5 s were probably associated with the surface or gut rather than being symbionts, because they always formed mixed colonies morphologically identical

Figure 8. Spiral-shaped bacteria (arrows) in a bacteriocyte from the connective tissue of an *Ophiocoma bollonsi* tube foot. HC, host cell; open arrows, host cell membrane.

Figure 9. Tightly kinked spiral form Type 2 SCB (arrow) from a tube foot of *Amphiura amokurae*. C, cuticle.

Figure 10. Type 3 SCB (arrows) from a tube foot of *Ophiomyxa brevirima*. SCB have numerous vacuoles (v). C, cuticle; HV vacuoles of host origin in sub-cuticular space.

Figure 11. Vacuolated Type 2 rods from a tube foot of *Amphipholis squamata*. Curved arrow indicates double membranes around SCB. C, cuticle; v, vacuoles.

to those from controls. No bacterial colonies of the type reported by Walker and Lesser (1989) were observed, even after 21 days. These results do not necessarily mean that the bacterium isolated by Walker and Lesser (1989) was not the symbiont. The SCB of *A. squamata* from the east coast of the United States could be different from the New Zealand or European symbionts and could either be resistant to isopropyl alcohol or have simpler culturing requirements. Successful isolation into culture of a bacterial symbiont from marine organisms is, however, very rare. Only the luminescent symbionts of some fish and squid (Hastings and Nealson, 1981; McFall-Ngai, 1994) have definitely been cultured, and these bacteria are commonly occurring, free-living forms. The problems of symbiont isolation are illustrated by a meticulous attempt by Wood and Kelly (1989) to culture chemoautotrophic bacteria from the bivalve *Thyasira flexuosa.* They isolated a sulfide-oxidizer of the genus *Thiobacillus* and proposed it as the symbiont of *T. flexuosa;* however, when genetic sequences of the cultured bacterium were compared to the symbionts, it was found that they were not the same (Marine Biological Association of the United Kingdom Annual Report 1991–1992).

As evidence that the bacteria they cultured were indeed SCB, Walker and Lesser (1989) produced polyclonal antibodies to whole bacterial cells, then used these antibodies to label sections of the host material. The SCB reacted with the polyclonal antibodies. Unfortunately, these authors did not try to determine the specificity of their antibodies to the cultured bacterial isolate by testing them against either a range of bacteria isolated from the gut and outer surfaces of *A. squamata,* or against other marine bacteria. It is therefore possible that these antibodies were not *Vibrio*-specific. Nor did these authors cross-react the antibodies with other *Vibrio* strains. It would not be surprising if SCB were related to the genus *Vibrio,* because unculturable symbionts from flashlight and angler fishes (Haygood and Distel, 1993) were shown to be related to vibrios. The polyclonal antibody results do show that the cultured bacterium shares an antigen with the SCB, but further investigations—preferably with monoclonal antibodies and *in situ* hybridization with species-specific rRNA probes (Distel *et al.,* 1991)—are needed to show that the cultured bacteria are genuinely the same as the SCB.

Table IV

Fisher's least-square difference table for comparisons of rods (Type 3 SCB) and spirals (Type 2 SCB) between Pseudechinus huttoni *(PH);* P. huttoni *juveniles (PHJ);* P. novaezealandiae *(PN), and* P. albocinctus *(PA); significance is at* $P < 0.05$

Comparison	Significance
PA rods > PN rods	0.0260
PH rods > PA rods	<0.0001
PHJ rods > PA rods	0.0017
PA spirals > PA rods	NS
PA rods > PN spirals	NS
PA rods > PH spirals	NS
PA rods > PHJ spirals	NS
PH rods > PN rods	<0.0001
PHJ rods > PN rods	<0.0001
PA spirals > PN rods	0.002
PN spirals > PN rods	NS
PH spirals > PN rods	NS
PHJ spirals > PN rods	NS
PH rods > PHJ rods	<0.0001
PH rods > PA spirals	<0.0001
PH rods > PN spirals	<0.0001
PH rods > PH spirals	<0.0001
PH rods > PHJ spirals	<0.0001
PHJ rods > PA spirals	NS
PHJ rods > PN spirals	0.0005
PHJ rods > PH spirals	<0.0001
PHJ rods > PHJ spirals	<0.0001
PA spirals > PN spirals	0.0486
PA spirals > PH spirals	0.0015
PA spirals > PHJ spirals	0.0066
PN spirals > PH spirals	NS
PN spirals > PHJ spirals	NS
PHJ spirals > PH spirals	NS

Table III

Bacterial counts per gram of tissue wet weight (WWT) and ash-free dry weight (AFDW)

Species	Weight of tissue (g)	Count from homogenate (standard deviation)	SCB g^{-1} WWT	SCB g^{-1} AFDW
Pseudechinus huttoni	0.958	37.0 (13.0)	2.19×10^8	4.48×10^9
P. albocinctus	0.986	16.8 (10.5)	9.96×10^7	2.17×10^9
P. novaezealandiae	0.608	6.37 (5.2)	3.77×10^7	8.41×10^8
P. huttoni juveniles	0.314	22.1 (12.4)	1.31×10^8	2.67×10^9
Asterodon miliaris	0.577	34.3 (19.6)	2.03×10^8	1.99×10^9
*Amphipholis squamata**	0.030	83.8 (14.0)	4.96×10^8	4.96×10^9
Ophiocoma bollonsi	0.849	6.9 (6.0)	4.09×10^7	4.41×10^8

The wet weight is an average per homogenate; the counts are an average of 20 fields of view and a total count of rods and spirals.

* AFDW conversion factor taken from Scottish west coast specimens (McKenzie and Kelly, 1994).

If SCB are unculturable, identifying the nature of their interaction with their hosts is more difficult. There has been a great deal of interest in associations between chemoautotrophic bacteria and various marine invertebrates. There is, however, no positive evidence suggesting that SCB are chemoautotrophs. Bacterial morphology sometimes provides clues to trophic biology (Berkeley, 1979), but the morphology of the SCB from the New Zealand echinoderms was not particularly informative. Type 2 SCB showed little internal specialization; even vacuoles were rare. The Type 3 SCB from the New Zealand species were more diverse. The profusion of vacuoles seen in the brittlestar *Ophiomyxa* and the holothurian *Stichopus mollis* may indicate that these bacteria are more unusual than other SCB so far observed. There was, however, no evidence of the specialized membrane stacks known from symbiotic methylotrophs (Fisher *et al.*, 1993) and nitrifiers (Stanier *et al.*, 1977). Nor was there any evidence of the sulfur storage observed in some sulfide-oxidizing symbionts (Southward, 1986). The lack of obvious morphological clues need not exclude chemoautotrophy. Some other sulfide-oxidizers, for example, do not store sulfur and they resemble SCB (Southward, 1986). Given the large number and diversity of host species, some SCB may yet prove to be chemoautotrophic.

Many investigators favor the suggestion that SCB metabolize dissolved organic material (DOM) (Holland and Nealson, 1978; Walker and Lesser, 1989), and some evidence supports this hypothesis. In *Amphipholis squamata*, SCB can take up dissolved amino acids, and this uptake precedes any translocation of synthesized proteins to the host (Walker and Lesser, 1989; Lesser and Walker, 1992). Host epidermal cells frequently phagocytose SCB (Walker and Lesser, 1989; Roberts *et al.*, 1991; McKenzie and Kelly, 1994). SCB could have a nutritive role if they used DOM as an energy source, then were "cropped" by the host through phagocytosis. It is more usual, however, for a host to benefit from the products of its symbionts than to ingest the bacteria themselves (Douglas, 1994), and it may be that only moribund SCB are phagocytosed. The division rate of the SCB is unknown—there are only estimates for standing crop—but given the frequency of observations of phagocytosis, it may be high. Lesser and Walker (1992) concluded, however, that the rate of DOM uptake was too low to provide a significant energy source to the host. They based this conclusion on a comparison between a symbiont-containing ophiuroid, *Amphipholis squamata*, and a species that lacked SCB. Unfortunately, the latter was *Ophiopholis aculeata*, a species that definitely possesses SCB, albeit in lower numbers than are found in *A. squamata* (McKenzie and Kelly, 1994). The role of SCB in DOM uptake has, therefore, still to be defined. Because of the density of symbionts observed in some species, the possible contribution of SCB to DOM uptake in echinoderms must be considered in investigations of growth and regeneration.

Although the bacteria seen in the bacteriocytes of *O. bollonsi* had a regular, "crimped" appearance when viewed with TEM, they are probably the same as the faintly fluorescing rods seen in the tube-foot squash preparations, the "crimped" shape being too small to be resolved with epifluorescence microscopy. These bacteria are not SCB, and both this species and *Ophiopteris antipodum* have been discounted from previous estimates of the numbers of species that, on the basis of epifluorescent evidence, are thought to harbor SCB (Kelly *et al.*, 1994). The bacteria counted in the homogenate of arm tissue from *Ophiocoma bollonsi* were probably also released from the bacteriocytes. The counts made from homogenates of *Ophiocoma bollonsi* (Table III) suggest that these bacteria are almost as abundant as SCB in other species. *Ophiopteris papillosa* from the northwestern coast of America is similar to *Ophiopteris antipodum* in that the rod-shaped bacteria seen with epifluorescence could not be found under the cuticle with TEM (McKenzie and Kelly, 1994). Further investigations may reveal bacteriocytes in these species also. Nothing is known of the biology of the bacteriocytes, but the host animals appear healthy. Large cells, filled with rod-shaped bacteria, have also been found in the crinoid *Calamocrinus diomedae* (Holland *et al.*, 1991). Similar structures have been found in other stalked crinoids (U. Welsh, pers. comm.) and in the comatulids *Antedon bifida* (Kelly and McKenzie, in press) and *A. petasus* (Heinzeller and Welsch, 1994). These bacteria may be a second type of symbiont, but they are also reminiscent of rickettsial infections of marine invertebrates (Sparks, 1985), which often have no obvious pathological effects on their hosts.

The SCB in New Zealand echinoderms are very similar to those in echinoderms from Europe and North America. Although some of the SCB have interesting morphologies that have not previously been encountered, almost all of the SCB from New Zealand species can be classified within two major types. The overall pattern of SCB distribution is similar to that in other echinoderm faunas (Kelly and McKenzie, in press) and no ecological trends in their distribution are obvious. The New Zealand study highlights both the restricted degree of variation in SCB morphology and the possibility that more than one type of SCB can occur within a single host species. It also strengthens the observation that closely related species are all likely to either have SCB or lack them (McKenzie and Kelly, 1994). The recorded densities of SCB indicate that they are potentially important to their hosts, although their exact functions are as yet unknown. None appear to be chemoautotrophs. Molecular techniques will be the best way to investigate the links between host phylogeny and SCB distribution. In the absence of isolated symbionts, experiments comparing nonsymbiotic species with ones con-

taining SCB may be the most productive means to explore trophic interactions between SCB and their hosts.

Acknowledgments

This work was partly funded by the Waitangi Fellowship to MSK from The Bank of Scotland and by Mobil North Sea Ltd. We thank the Directors of the Portobello and Dunstaffnage Marine Laboratories for the use of the facilities; and Mr. Alan Mitchell and the staff at the South Campus EM Unit, University of Otago; the crew of the R. V. *Munida;* and Dr. Mark James for their technical support.

Literature Cited

Berkeley, R. C. W. 1979. Structure and classification of prokaryotic micro-organisms. Pp. 135–175 in L. E. Hawker and A. H. Linton, eds. *Micro-organisms: Function, Form and Environment.* (2nd Edition). Arnold, London.

Bosch, C. 1976. Sur un noveau type de symbiose chez la Bonellie (*Bonellia viridis*, Echiurien). *C. R. Acad. Sci. Paris* **282:** 2179–2182.

Bosch, I. 1992. Widespread symbiosis between bacteria and sea star larvae in epipelagic regions of the North Atlantic. *Mar. Biol.* **114:** 495–502.

Buchner, P. 1966. *Endosymbiosis of Animals with Plant Microorganisms.* Wiley, New York. 909 pp.

Cameron, R. A., and N. D. Holland. 1983. Electron microscopy of extracellular materials during the development of a seastar, *Patiria miniata* (Echinodermata; Asteroidea). *Cell Tissue Res.* **234:** 193–200.

Cavanaugh, C. M., S. L. Gardiner, M. L. S. Jones, H. W. Jannasch, and J. B. Waterbury. 1981. Prokaryotic cells in the hydrothermal vent tube worm *Riftia pachyptila;* possible chemoautotrophic symbionts. *Science* **213:** 340–342.

Distel, D. L., E. F. DeLong, and J. B. Waterbury. 1991. Phylogenetic characterisation and in situ localization of the bacterial symbiont of shipworms (Teredinidae: Bivalvia) by using 16S rRNA sequence analysis and oligodeoxynucleotide probe hybridization. *Appl. Environ. Microbiol.* **57:** 2376–2382.

Douglas, A. E. 1994. *Symbiotic Interactions.* Oxford University Press, Oxford. 148 pp.

Féral, J. P. 1980. Cuticule et bacteries associées aux épidermes digestif et tégumentaire de *Leptosynapta galliennei* (Herapath) (Holothuroidea: Apoda)—premières données. Pp. 285–290 in *Echinoderms Present and Past.* M. Jangoux., ed. Balkema, Rotterdam.

Fell, H. B. 1962. *Native Sea Stars.* A. H. and A. W. Reed, Wellington.

Fenchel, T., T. Perry, and A. Thane. 1977. Anaerobiosis and symbiosis with bacteria in free-living ciliates. *J. Protozool.* **24:** 154–163.

Fisher, C. R., J. M. Brooks, J. S. Vodenichar, J. M. Zande, J. J. Childress, and R. A. Burke, Jr. 1993. The co-occurrence of methanotrophic and chemoautotrophic sulfur-oxidising bacterial symbionts in a deep-sea mussel. *Mar. Ecol.* **14:** 277–289.

Giere, O. 1981. The gutless marine oligochaete *Phallodrilus leukodermatus*. Structural studies on an aberrant tubificid associated with bacteria. *Mar. Ecol. Prog. Ser.* **5:** 353–357.

Hastings, J. W., and K. H. Nealson. 1981. The symbiotic luminous bacteria. Pp. 1332–1345 in *The Prokaryotes—A Handbook of Habitats, Isolation and Identification of Bacteria.* M. P. Starr *et al.*, eds. Springer-Verlag, Berlin.

Haszprunar, G., K. Schaefer, A. Waren, and S. Hain. 1995. Bacterial symbionts in the epidermis of an Antarctic neopilinid limpet (Mollusca, Monoplacophora). *Proc. Roy. Soc. Lond.*, in press.

Hausmann, K. 1982. Elektronenmikroskopische Untersuchungen an *Anaitides mucosa* (Annelida Polychaeta). Cuticula and Cilien, Schleimzellen und Schleimextrusion. *Helgol. wiss. Meeresunters* **35:** 79–96.

Haygood, M. G., and D. L. Distel. 1993. Bioluminescent symbionts of flashlight fishes and deep-sea anglerfishes from unique lineages related to the genus *Vibrio*. *Nature* **363:** 154–156.

Heinzeller, T., and U. Welsch. 1994. Crinoidea. Pp. 9–148 in *Microscopic Anatomy of Invertebrates, Volume 14: Echinodermata.* Wiley-Liss, New York.

Hobbie, R. J., R. J. Daley, and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* **33:** 1225–1228.

Holland, N. D., and K. H. Nealson. 1978. The fine structure of the echinoderm cuticle and subcuticular bacteria of echinoderms. *Acta Zool. (Stockh.)* **59:** 169–185.

Holland, N. D., J. C. Grimmer, and K. Wiegmann. 1991. The structure of the sea lily *Calamocrinus diomedae* with special reference to the articulations, skeletal microstructure, axial organs and stalk tissues (Crinoida, Millericrinida). *Zoomorphology* **110:** 115–132.

Houk, E. J., and G. W. Griffiths. 1980. Intracellular symbiotes of the Homoptera. *Ann. Rev. Entomol.* **25:** 161–187.

Kelly, M. S., and J. D. McKenzie. 1992. The quantification of subcuticular bacteria in echinoderms. Pp. 225–228 in *Echinoderm Research* 1991, L. Scalera-Liaci and C. Canicattì, eds. Balkema, Rotterdam.

Kelly, M. S., J. D. McKenzie, and M. F. Barker. 1994. Sub-cuticular bacteria: their incidence in the echinoderms of the British Isles and New Zealand. Pp. 33–38 in *Echinoderms. Dijon*, B. David *et al.*, eds. Balkema, Rotterdam.

Kelly, M. S., and J. D. McKenzie. 1995. A survey of the occurrence and morphology of sub-cuticular bacteria in shelf echinoderms from the north-east Atlantic. *Mar. Biol.* in press.

Lesser, M. P., and R. P. Blakemore. 1990. Description of a novel symbiotic bacterium from the brittlestar *Amphipholis squamata*. *Appl. Environ. Microbiol.* **56:** 2436–2440.

Lesser, M. P., and C. W. Walker. 1992. Comparative study of the uptake of dissolved amino acids in sympatric brittlestars with and without endosymbiotic bacteria. *Comp. Biochem. Physiol.* **101B:** 217–223.

Lutaud, G. 1969. La nature des corps funiculaires des cellularines bryozoaires chilostomes. *Arch. Zool. Exp. Gen.* **110:** 2–30.

McFall-Ngai, M. J. 1994. Animal-bacterial interactions in the early life-history of marine invertebrates: the *Euprymna scolopes/Vibrio fischeria* symbiosis. *Am. Zool.* **34:** 554–561.

McKenzie, J. D. 1987. The ultrastructure of the tentacles of eleven species of dendrochirote holothurians studied with special reference to the surface coats and papillae. *Cell Tissue Res.* **248:** 187–199.

McKenzie, J. D. 1992. Comparative morphology of crinoid tube feet. Pp. 73–79 in *Echinoderm Research 1991*, L. Scalera-Liaci and C. Cancatti, eds. Balkema, Rotterdam.

McKenzie, J. D., and M. S. Kelly. 1994. A comparative study of subcuticular bacteria in brittlestars (Echinodermata: Ophiuroidea). *Mar. Biol.* **120:** 65–80.

Mackie, G. O., and Q. Bone. 1978. Luminescence and associated effector activity in *Pyrosoma* (Tunicata: Pyrosomida). *Proc. Roy. Soc. Lond. B* **202:** 483–495.

Margulis, L. 1981. *Symbiosis in Cell Evolution.* Freeman, San Francisco. P. 418.

Menon, J. G., and A. J. Arp. 1993. The integument of the marine echiuran worm *Urechis caupo*. *Biol. Bull.* **185:** 440–454.

Muscatine, L., P. Falkowski, J. Porter, and Z. Dubinsky. 1984. Fate of photosynthetically-fixed carbon in light and shade-adapted colonies of the symbiotic coral *Stylophora pistillata*. *Proc. Roy. Soc. Lond. B* **222:** 181–202.

Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. *Physiology of the Bacterial Cell: A Molecular Approach.* Sinauer, Sunderland, MA.

Ott, J., G. Rieger, R. Rieger, and F. Enderes. 1982. New mouthless interstitial worms from the sulphide system: symbiosis with prokaryotes. *Mar. Ecol.* **3:** 313–333.

Palinesar, E. E., W. R. Jones, J. S. Palinesar, M. A. Glogowski, and J. L. Mastro. 1989. Bacterial aggregates within the epidermis of the sea anemone *Aiptasia pallida. Biol. Bull.* **177:** 130–140.

Powell, M. A., and G. N. Somero. 1983. Blood components prevent blood poisoning of respiration of the hydrothermal vent tube worm *Riftia pachyptila. Science* **219:** 297–299.

Roberts, D., D. S. M. Billet, G. McCartney, and G. E. Hayes. 1991. Procaryotes on the tentacles of deep-sea holothurians: a novel form of dietary supplementation. *Limnol. Oceanogr.* **36:** 1447–1452.

Saffo, M. B. 1990. Symbiosis within a symbiosis: intracellular bacteria within the endosymbiotic protist *Nephromyces. Mar. Biol.* **107:** 291–296.

Smith, D. C., and A. E. Douglas. 1987. *The Biology of Symbiosis.* Arnold, London.

Sparks, A. K. 1985. *Synopsis of Invertebrate Pathology.* Elsevier, Amsterdam. P. 423.

Southward, E. C. 1986. Gill symbionts in thyasirids and other bivalve molluscs. *J. Mar. Biol. Ass. U. K.* **66:** 889–914.

Souza Santos, H., and W. S. Sasso. 1970. Ultrastructural and histochemical studies on the epithelium revestment layer in the tube feet of the starfish *Asterina stellifera. J. Morphol.* **130:** 287–296.

Stanier, R. Y., E. A. Adelberg, and J. L. Ingraham. 1977. *General Microbiology.* MacMillan, London.

Vacelet, J., and C. Donadey. 1977. Electron microscopy study of the association between some sponges and bacteria. *J. Exp. Mar. Biol. Ecol.* **30:** 301–314.

Walker, C. W., and M. P. Lesser. 1989. Nutrition and development of brooded embryos in the brittlestar *Amphipholis squamata:* do endosymbiotic bacteria play a role. *Mar. Biol.* **103:** 519–530.

Welsch, U. 1984. Hemichordata. Pp. 791–799 in J. Bereiter-Hann, A. G. Matoltsy, and K. S. Richards, eds. *Biology of the Integument, Vol 1.* Springer Verlag, Berlin.

Wood, A. P., and D. P. Kelly. 1989. Isolation and physiological characterisation of *Thiobacillus thyasiris* sp. nov., a novel marine facultative autotroph and the putative symbiont of *Thyasira flexuosa. Arch. Microbiol.* **152:** 160–166.